(free) end to a microscopic bead. The DNA tether restricts the Brownian motion of the bead and the average amplitude of excursions of the bead from the anchor point indicates the DNA tether length. Excursions of the beads depend on the physical properties of the DNA-bead system, and the physical and chemical properties of the surrounding solution. Previously published TPM calibrations, for a range of DNA lengths and 200 to 970 nm diameter beads showed that larger beads increase excursions and comparatively more time is necessary for a bead to fully explore the available volume and report the maximum excursion for a longer as opposed to a shorter DNA molecule. Others have shown that magnesium ions reduce the excursions and increased viscosity slows the diffusion of an 80 nm gold bead tethered by a single length of DNA. New experiments with 320 and 480 nm diameter beads and DNA of several lengths show increasing viscosity increases the time required to report the maximum average excursion. Interpreting these results based on the dynamics of the viscous solvent-DNA-bead system highlights important experimental details for TPM measurements of DNA protein interactions and indicates how the kinetics of topological rearrangements might be impacted by increased viscosity environments such as cytoplasm.

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Application of 3-D Prismatic Optical Tracking to Single-Molecule Optical Tweezers
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We have developed a new method for tracking single particles in three dimensions under a conventional microscope by dividing light in two components using a wedge prism. This method, three-dimensional prismatic optical tracking, termed ***tPOT***, is here combined with optical tweezers to quantify molecular scale 3-D force in motor proteins. Precise calibration of trapping force to bead displacement with sub nanometer scale enables us to determine the force exerted not only in *xy*-plane direction but also along optical axis (*z*-axis). With this experimental set-up, we applied force to rotary motor F_1-ATPase that showed rotation on a glass surface. Our research group previously used the procedure in which the objective was displaced to impose the load on single molecules held between the trapped bead and the glass, which frequently caused the measurement error because of drifting motions of the specimen. This method has been modified as follows; 1) the trap center is displaced with time by moving the focusing lens along *z*-axis; 2) spring constant of the trap is increased via the adjustment of the laser power. In either case positions of the stage and the objective were fixed and thus repetitive measurements were reliably performed. The rupture force of the bead from the glass surface, which presumably reflects the interaction between the shaft and the cylinder of F_1-ATPase, distributed up to 40 pN and showed 19 pN on the average. The wide distribution of the force may be originated from different chemical states of the cylinder when the rupture occurs. We also demonstrate various applications of ***tPOT***, such as corkscrewing motions of linear molecular motors or beating motions of ciliary axonemes.

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Molecular Mechanotransduction in Human CD4
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HIV-1 infection initiates when the viral envelope glycoprotein gp120 interacts with two receptors on the surface of the T lymphocytes, CD4 and a chemokine co-receptor (CCR5 or CXCR4). Upon binding, a cascade of conformational changes in CD4 triggers viral fusion to the cell membrane. However, the key factors that originate the structural alterations in CD4 are unclear. Here, we use single-molecule force spectroscopy to study the mechanochemical properties of the two more external domains of human CD4 (D1 and D2). The application of mechanical forces to the CD4 domains reveals that they can be unfolded in a time-dependent manner within a biological range of forces. Similarly to other tandem repeats proteins such as titin or tenascin, mechanical unfolding of CD4 modules might be an important process occurring in vivo acting as a shock absorber. This phenomenon would help to prevent viral detachment. Furthermore, we show that mechanical exposition of internal disulfide bonds in CD4 is required for redox regulation by thioredoxin enzymes, a process that has been suggested to occur during viral infection. In addition, we perform numerical calculations using suitable models for polymer elasticity to correlate viral infectivity (Freeman et al. Structure 18(12):163241) with mechanical extensibility of CD4 modules. The role of mechanical forces in HIV-1 infection is yet to be considered, but it might represent a new view to better understand viral infection.

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Single-Molecule Optical Trap Study of Human CCR5 mRNA Structure
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Tertiary structure of mRNA can regulate translation by the ribosome. Specifically, potential pseudoknot structures are found in many genes which may stimulate programmed −1 ribosomal frame shifting. We conducted single-molecule optical trapping experiments on CCR5 mRNA, which encodes the co-receptor for HIV-1. We found that individual molecules of CCR5 mRNA will randomly fold into several unique conformations when stretched repeatedly, one of these being consistent with a proposed pseudoknot structure. Other conformations have higher energies than would be expected for duplex RNA and may be explained by tertiary RNA structure. A new analysis technique was applied, where we consider the change in energy per distance (dE/dx) of the molecule. This method of analysis provides a useful means to classify the many conformations the mRNA adopts as well as providing unique insights into the structure of CCR5 mRNA.

1956-Pos Board B726
Fluorogen Activating Peptides that Enhance Photostability through Encapsulation of the Fluorogen
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Biological imaging has profited from the development of a variety of fluorescent probes such as organic dyes, quantum dots and fluorescent proteins. More recently, Fluorogen Activating Peptides (FAP) have been developed through directed evolution. These FAPs bind non-covalently to an otherwise non-fluorescent molecule (fluorogen) to form fluorescent complexes. FAPs have been successfully employed in various live-cell imaging applications in the past two years. For live-cell and single molecule imaging applications, photostability is a key requirement for any fluorescent probe. FAPs have been shown to exhibit high photostability in cell-imaging applications. An understanding of the mechanism underlying the photostability of these FAPs will help us in future to develop FAP clones with higher photostability. In this study, we have characterized the photobleaching behavior of two Malachite green (MG) binding FAP clones: dL5NP138 and HL4. These studies have been performed at a single molecule level. These studies have shown FAPs to be more photostable compared to a widely used single molecule dye, Cy5, at a single molecule condition. Additionally we have exposed FAPs and the Green fluorescent protein to vigorous oxidizing conditions to assess the encapsulation of the chromophore in the protein environment.
Our studies show that FAPs protect MG (the fluorogen) from external environments that are rich in reactive oxygen species. The same behavior was observed by GFP compared to a synthetic soluble GFP chromophore. Using single molecule approaches in the presence of excess dye, we have demonstrated that these MG-binding FAPs are photostable through encapsulation of the fluorogen, and not by exchange of the bleached fluorogen. This mechanism provides MG-FAP complexs that can be used for single molecule detection over periods greater than Cy5 labels, yet with the convenience of genetically encoded reporters such as GFP.

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Single Molecule Counting using STORM/PALM
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Single-molecule counting using STORM/PALM
During the last decade, several super-resolution microscopy techniques have been developed that enable the circumvention or even the breaking of Abbe's diffraction limit. One approach of achieving super-resolution is to control the emission state of individual fluorophores in time and the concomitant ability to localize active emitters with up to nanometer accuracy. Two of the most commonly implemented super-resolution techniques are stochastic optical reconstruction microscopy (STORM) and photo-activated localization microscopy (PALM). Each technique uses the localization of individual emitters to improve resolution as has been demonstrated with numerous intracellular structures. Beyond that, these techniques open the possibility of counting on a single molecule level. However, the ability to count is fundamentally limited by the fluorophores used for these techniques which blink many times before photobleaching. A typical STORM dye like Cy5 can cycle between tens and hundreds of times between a dark and a fluorescent state before

photobleaching, thus leading to a set of localizations scattered around the true position. Whereas this property does not affect the reconstruction of images of extended objects like intracellular filaments or compartments, it severely influences the interpretation and quantification of objects for which the exact stoichiometry can be important like for membrane protein aggregates. To resolve this issue, we have implemented an algorithm that uses the spatial and temporal information of fluorophore localizations from STORM/PALM experiments to obtain a quantitative picture of the underlying molecule distribution. Our algorithm reliably operates on artificial data as well as on experimental data from biological constructs with a well-defined number of attached fluorophores.

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Regulating Bacterial Cytokinesis: A Super-Resolution Study of ZapA and ZapB

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E. coli cell division is achieved by the concerted effort of at least 13 essential proteins that assemble at midcell in a cell-cycle dependent manner to form a macromolecular structure (divisome) capable of generating septal force. Central to divisome assembly is the initial formation of the Z-ring, a dynamic, cytokinetic suprastructure of the FtsZ protein, the prokaryotic tubulin homolog widely conserved in bacteria. Being the first division protein to localize precisely to midcell, spatial and temporal regulation of FtsZ polymerization is of critical importance to efficient proliferation.

From conventional fluorescence microscopy, the Z-ring has long been regarded as a closed-ring that circumscribes the cell. However, in vitro studies illustrated FtsZ's tendency to self-assemble into short, single-stranded protofilaments that further coalesce into multi-stranded rings and helices under molecular crowding conditions. Recently, our group has shown that the relevant in vivo structure of FtsZ is characterized by an irregular, discontinuous arrangement of overlapping protofilaments, observed in a closed ring as well as a compressed helical conformation. We believe that an equilibrium exists between the helix and ring conformations and therefore factors promoting the transition from helix to ring may serve as critical regulatory elements or pathways.

ZapA and ZapB are both non-essential, cytoplasmic proteins that associate directly with FtsZ early during division. Although their null mutants have little observable cytokinetic defect, both have a pronounced prevalence of FtsZ helical conformations, indicating their presence favors ring formation. In this study, we employ super-resolution imaging to characterize the relevant in vivo arrangements of ZapA and ZapB, as well as their relative localization with respect to FtsZ using two-color imaging methods. Lastly, by characterizing the helical conformation the Z-ring in the Zap-null mutants, we gain insight to their potential regulatory mechanism.

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Corral Trapping of Single Molecules in Solution: Theory and Applications

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One of the great outstanding challenges for the fabrication of nanosensors and nanodevices that will drive future technologies and enable the planned assembly of molecular-scale devices is the controlled manipulation of nanoscale objects, and particularly of single molecules. Any such manipulation is preceded by an efficient, reliable method for confining (trapping) an object on demand, which has remained a formidable task in the case of single molecules.

We have successfully trapped single molecule ions and other charged particles in aqueous electrolytes using a purely electrostatic setup, demonstrating stable and reversible confinement of single and multiple particles to nanoscale dimensions over extended periods of time.

Here, we will present some of the experimental results and discuss potential mechanisms for corral trapping. Electrokinetic phenomena such as electrohydrodynamic flow, electroosmotic flow, or dielectrophoresis are known to occur near a charged metal electrode; however, we attribute corral trapping to direct charge-field interactions, i.e. and electrostatic or electrophoretic mechanism, which seems consistent with all experimental observations. Theoretical modeling of the entire setup using the finite element method will be discussed, and the limitations for applications in SNP detection and water treatment technologies will be explored.

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A Rejection Algorithm Essential for Quantitative Analysis in Single Molecule Super Resolution Microscopy

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Single molecule localization based super-resolution (SM-SR) imaging techniques require robust identification and accurate localization of single molecules to yield reliable emitter position estimates for further quantitative analysis. One of the key steps of SM-SR analysis is to clearly identify isolated, single emitters such that obtained localizations relate to actual single molecule locations, and are not influenced by signal from nearby or overlapping fluorophores. We refer to the process of identifying and discarding sub-regions that contain overlapping fluorophores as the rejection algorithm.

We show that even at an optimal active emitter density, more than 50% of the emitters cannot be isolated such to give an unbiased estimate of position. Furthermore, it is possible that even for the case of isolated single emitters, the fitting routine may not converge and thus provides incorrect localizations. A robust, statistically rigorous rejection algorithm that compares the fitted model to the data is necessary to avoid incorrect or biased localizations and is essential for the success of quantitative analysis.

Here, we show that the Log-likelihood Ratio (LLR) is a preferred metric for rejection algorithms and that p-values can be calculated and used for statistical significance tests when the distribution of the LLR under low signal conditions is correctly modeled. We compare the LLR with other commonly used rejection algorithms such as the elliptical or sum of squared error tests. As a demonstration, we show the improved resolution in reconstructed super-resolution images when using the LLR rejection algorithm as compared with those generated using other rejection algorithms.

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Dynamics of Nuclear Protein Exploration Revealed by Intracellular Single Particle Tracking PALM

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Cellular regulation of eukaryotic cells involves molecular interactions of factors diffusing within the cellular volume. Understanding the gene expression regulation requires thus elucidating the spatio-temporal dynamics of intranuclear proteins at the single molecule level. However, live cell imaging of single molecules in eukaryotic cells has remained mostly restricted to processes occurring in the plasma membrane, readily accessible by TIRF microscopy as opposed to intra-nuclear processes.

We report an intracellular single particle tracking method using photoactivated localization microscopy that enables the study of protein dynamics inside live eukaryotic cells. So far single particle tracking PALM (sptPALM) (Manley et al, 2008) has been restricted to cellular systems for which imaging can be performed using total internal reflection microscopy (TIRF), and believed to be limited to slow diffusing systems (~ 0.1 um2/s). Here we demonstrate an approach that reduces the background of out-of-focus fluorophores by a tight control of the photoactivation, thus allowing the detection and characterization of single protein dynamics directly in the nucleus of living cells.

Applying this method to several nuclear proteins, we captured a wide range of diffusive behaviors from very rapid diffusion (> 10 μm2/s) to bound chromatin associated states (< 0.1 μm2/s). We measured the single molecule dynamics for a diverse set of proteins, from free fluorophores (Dendra2) with no known interactions in the nucleoplasm, to DNA binding (c-Myc), RNA binding (Fibrillarin), and protein-protein interacting complexes (p-TEFb). We observe that, overall, nuclear exploration is not governed by a unique nucleoplasmic geometry but rather a protein-specific variable. Our approach provides a versatile tool for single molecule in vivo studies in eukaryotes.

References

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1962-Pos Board B732

New Tool for Single Molecule Manipulation: Optical Pushing

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